

Inhibition of Processing of Plant N-Linked Oligosaccharides by Castanospermine

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Castanospermine (1,6,7,8-tetrahydroxyoctahydroindolizine) is a plant alkaloid that inhibits lysosomal α - and β -glucosidase. It also inhibits processing of influenza viral glycoproteins by inhibiting glucosidase I and leads to altered glycoproteins with Glc₃Man₇GlcNAc₂ structures. Castanospermine was tested as an inhibitor of glycoprotein processing in suspension-cultured soybean cells. Soybean cells were pulse-labeled with [2-³H]mannose and chased for varying periods in unlabeled medium. In normal cells, the initial glycopeptides contained oligosaccharides having Glc₃Man₉GlcNAc₂ to Glc₁Man₉GlcNAc₂ structures and these were trimmed during the chase to Man₉GlcNAc₂ to Man₇GlcNAc₂ structures. In the presence of castanospermine, no trimming of glucose residues occurred although some mannose residues were apparently still removed. Thus, the major oligosaccharide in the glycopeptides of castanospermine-incubated cells after a 90-min chase was a Glc₃Man₇GlcNAc₂ structure. Smaller amounts of Glc₃Man₆GlcNAc₂ and Glc₃Man₅GlcNAc₂ were also identified. Thus, in plant cells, castanospermine also prevents the removal of the outermost glucose residue.

The biosynthesis of the oligosaccharide portion of the N-linked glycoproteins of plant and animal cells involves the participation of lipid-linked saccharide intermediates (1, 2). This pathway ultimately leads to the formation of a Glc₃Man₉GlcNAc₂²-pyrophosphoryl-dolichol which was initially isolated and characterized from animal cells (3-5). A glucose-containing oligosaccharide-lipid was also isolated from several plant systems (6, 7), and our studies on the characterization of this Glc₃Man₉GlcNAc₂-lipid from suspension-cultured soybean cells (8) suggested that the plant oligosaccharide is probably similar, if not identical, to that from animal cells. In animals, following

transfer of the Glc₃Man₉GlcNAc₂ to protein, the newly formed glycoprotein undergoes a number of processing reactions that result in the removal of all three glucose residues and some of the mannose residues (reviewed in Ref. (9)). We recently demonstrated that the N-linked oligosaccharides of soybean cells also undergo similar kinds of processing reactions (10). Thus, although plants do not contain the typical types of complex oligosaccharides, their N-linked oligosaccharides are trimmed by the removal of all three glucose residues and some of the mannose residues to form oligosaccharides having 5, 6, 7, or 8 mannose units. In addition, both glucosidase and mannosidase activities were detected in cell-free extracts of soybean cells.

Since processing reactions are important for determining the nature of the final oligosaccharide structure and also may play a key role in receptor activity and recog-

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² Abbreviations used: Glc, glucose; Man, mannose; GlcNAc, N-acetylglucosamine.

nitron reactions, it was of major interest to examine the effect of processing inhibitors on oligosaccharide structures in soybean cells. Castanospermine (1,6,7,8-tetrahydroxyoctahydroindolizine) is an indolizidine alkaloid that is found in the seeds of the Australian plant, *Castanospermum australe* (11). Castanospermine was found to be a potent inhibitor of lysosomal α - and β -glucosidases (12), and also inhibited the processing of the oligosaccharide chains of the influenza viral hemagglutinin (13). The viral hemagglutinin produced in the presence of castanospermine contained mostly a Glc₃Man₇GlcNAc₂ structure, in keeping with *in vitro* studies showing that this alkaloid inhibited glucosidase I. In this report, we show that castanospermine also inhibits processing of the oligosaccharide chains in suspension-cultured soybean cells. In this case, the major oligosaccharides isolated from the glycopeptides were partially characterized as Glc₃Man₇GlcNAc₂, Glc₃Man₆GlcNAc₂, and Glc₃Man₅GlcNAc₂.

EXPERIMENTAL PROCEDURES

Growth of cells and incorporation of isotopes. Soybean cells (*Glycine max* L.) were cultured in Erlenmeyer flasks as previously described (10). Ten-day-old cells were harvested by filtration and washed with sucrose-free medium, and 30 g of cells was placed in 50 ml of sucrose-free medium in a 250-ml Erlenmeyer flask. Thirty microcuries of [2-³H]mannose were added to these flasks and they were incubated at 30°C for various times.

In some experiments, the cells were pulse-labeled with isotope for 15 min and then incubated in the absence of isotope for varying periods of time (chase). In such an experiment, 30 g of cells were incubated for 30 min in 30 ml of sucrose-free medium either in the presence or absence of castanospermine. Then, 200 μ Ci of [2-³H]mannose was added, and the incubation was continued for another 15 min. At the end of this time, the cells were harvested and washed on a Büchner funnel. The cells were resuspended in the fresh medium (containing sucrose, 10 mM mannose, and castanospermine where indicated) and incubated at 29°C. Five milliliters of cell suspension was removed at the indicated times.

Preparation of glycopeptides. The soybean cells harvested at various times were extracted with various CHCl₃:CH₃OH:H₂O mixtures to obtain the lipid-linked saccharides (8). The residue remaining after these extractions was placed in 20 mM Tris-HCl buffer

containing 10 mM CaCl₂. Five milligrams of Pronase was added for each 50 mg of residue and the mixture was incubated at 37°C for 24 h under a toluene atmosphere. Another 5 mg of Pronase was added after 24 h, and another at 48 h. After 72 h of incubation, ethanol was added to a final volume of 60% and insoluble material was removed by centrifugation. The supernatant fraction containing the glycopeptides was concentrated to a small volume and subjected to gel filtration on Bio-Gel P-4.

Chromatographic procedures and enzymatic digestions. Oligosaccharides and glycopeptides were separated on a 1.5 \times 150-cm column of Bio-Gel P-4 (200–400 mesh) equilibrated and run in 0.1 M acetic acid. The isolated glycopeptides were digested with various enzymes in order to assist in their characterization. Purified glycopeptides were treated with 10 mU of endoglucosaminidase H in 0.1 ml 50 mM sodium citrate, pH 6.0, at 37°C. After 24 h of incubation, another 10 mU was added. Reactions were run under a toluene atmosphere. Oligosaccharides were incubated with 0.5 units of jack bean α -mannosidase in 0.1 ml of 100 mM sodium acetate buffer, pH 5.0, containing 0.4 mM ZnCl₂ under a toluene atmosphere. After 24 h, another 0.5 units of enzyme was added. Products of the above reactions were analyzed on the Bio-Gel P-4 columns.

Methylation analysis of oligosaccharides. Methylation of the oligosaccharide was performed as described by Hakomori (14) and as modified by Sanford and Conrad (15). The lyophilized samples were dissolved in 2 ml of dimethylsulfoxide under N₂ and sonicated with methylsulfinylcarbanion at 50°C for 5 h. The mixture was chilled and 2 ml of CH₃I were added. The sample was again sonicated for 2 h at 4°C and another addition of 2 ml of CH₃I was made after 1 h. After standing at room temperature overnight, the sample was passed through a column of Sephadex LH-20, equilibrated and run in 80% CH₃OH, to remove dimethylsulfoxide, methylsulfinylcarbanion, and other salts. The eluate from the column was hydrolyzed in 2 N H₂SO₄ at 100°C for 4 h in screw-capped tubes. After neutralization with Ba(OH)₂, the methylated sugars were identified by thin-layer chromatography (8).

Materials. [2-³H]Mannose (16 Ci/mmol) and [2-³H]glucose (10 Ci/mmol) were purchased from Amersham Laboratories. Bio-Gel P-4 (200–400 mesh and –400 mesh) was from BioRad Laboratories. Endoglucosaminidase H was from Health Research, Inc., Albany and jack bean α -mannosidase was from Sigma Chemical Company. Various oligosaccharide standards were kindly provided by Dr. S. C. Hubbard and Dr. P. W. Robbins, Massachusetts Institute of Technology, and Dr. M. Reitman and Dr. S. Kornfeld, Washington University School of Medicine. Castanospermine (1,6,7,8-tetrahydroxyoctahydroindolizine) was isolated in 0.3% yield from mature seeds of *C. australe* by extraction with water and purification by ion exchange chromatography on Dowex 50W-X-8 (11).

RESULTS

In order to examine the normal processing of the oligosaccharide chains of the N-linked glycoproteins, suspension-cultured soybean cells were pulse-labeled with [^3H]mannose for 15 min, and the label was chased by placing the cells in complete medium without isotope for various times up to 2 h. At the times shown in figures, an aliquot of the cell suspension was harvested and, after extraction of lipids, digested exhaustively with Pronase to release glycopeptides. The glycopeptides were then examined by gel filtration on columns of Bio-Gel P-4. Figure 1 shows the glycopeptide profiles obtained on these columns at various times of chase. At the start of the chase (0 time), the radioactivity was found in a rather broad peak that eluted mostly between the standards $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ (arrow corresponding to 12) and $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$ (arrow corresponding to 10). However, with increasing times of chase, this radioactivity continuously shifted to later and later fractions indicating that the glycopeptides were becoming smaller in size. Thus, after 60 min of chase, the major peak of radioactivity corresponded to the $\text{Man}_9\text{GlcNAc}_2$ standard (arrow corresponding to 9), while at 120 min, the major radioactive peak corresponded to $\text{Man}_8\text{GlcNAc}_2$. Since these glycopeptides had been prepared by exhaustive digestion with the proteolytic enzyme Pronase, it seemed likely that the peptide portion was similar in all cases and that these alterations in size were due to processing of the oligosaccharide. Previous studies showed that the major oligosaccharides present at 0 time were mostly $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$, $\text{Glc}_2\text{Man}_9\text{GlcNAc}_2$, and $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$, whereas those found after 2 h of chase were high-mannose chains containing 7, 8, or 9 mannose residues (i.e., $\text{Man}_9\text{GlcNAc}_2$, $\text{Man}_8\text{GlcNAc}_2$, and $\text{Man}_7\text{GlcNAc}_2$ (10)). In Fig. 1, there is a small peak of radioactivity (fractions 105–112) that precedes the main peak of radioactivity. This peak has not been identified at this time. However, since this material is not susceptible to endoglucosaminidase H nor to α -mannosidase, it may be a mannose-containing polysaccharide. Thus, plant cells frequently contain

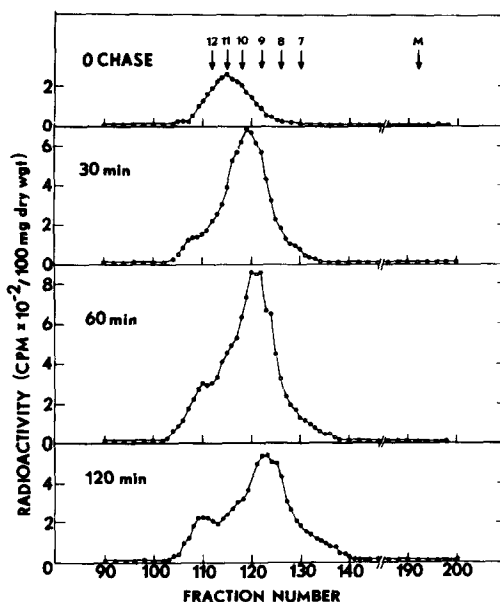


FIG. 1. Processing of N-linked oligosaccharides in normal soybean cells. Cells were pulse-labeled with [^3H]mannose for 15 min and then placed in unlabeled medium. At the times shown, cells were harvested, worked well with buffer, and extracted with $\text{CHCl}_3:\text{CH}_3\text{OH}$ to isolate the lipid-linked saccharides. The residue was digested exhaustively with Pronase and the liberated glycopeptides were isolated by gel filtration on Bio-Gel P-4. Aliquots of each fraction were removed for the determination of radioactivity. Arrows indicate migration of standards corresponding to $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ (12), $\text{Glc}_2\text{Man}_9\text{GlcNAc}_2$ (11), $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$ (10), $\text{Man}_9\text{GlcNAc}_2$ (9), etc.

β -mannans, glucomannans, or galactomannans (16).

Since the processing of plant glycoproteins involves the removal of all three glucose residues and a number of mannose residues, it was of interest to examine the effect of processing inhibitors in this system. Castanospermine is an indolizidine alkaloid that has been shown to inhibit lysosomal α - and β -glucosidase (12), and to prevent the processing of the influenza viral hemagglutinin by inhibiting glucosidase I (13). Thus, the effect of this alkaloid on glycoprotein processing in soybean cells was examined. Soybean cells were placed in the presence of 30 $\mu\text{g}/\text{ml}$ of castanospermine and labeled for 15 min with [^3H]mannose. At the end of this time, the cells were harvested by filtration,

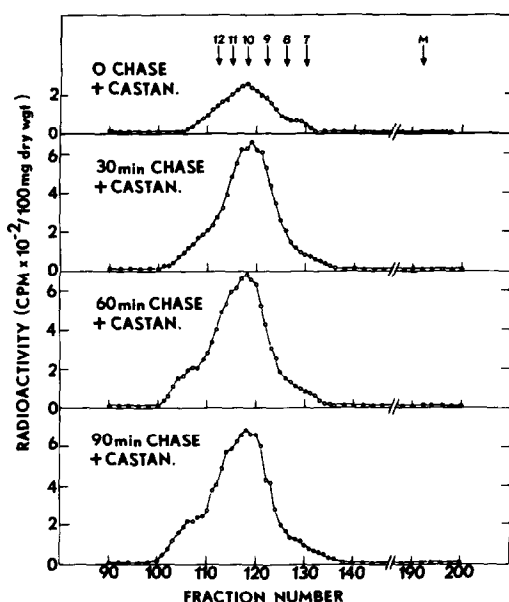


FIG. 2. Effect of castanospermine on glycoprotein processing by soybean cells. Soybean cells were placed in 30 μ g/ml of castanospermine and pulse-labeled for 15 min with [2- 3 H]mannose. Cells were then transferred to unlabeled medium containing castanospermine and incubated for the times shown. Glycopeptides were prepared as indicated in Fig. 1 and were isolated on Bio-Gel P-4. Standards are as indicated in Fig. 1.

and resuspended in fresh medium without isotope but containing castanospermine at 30 μ g/ml. At the times shown in Fig. 2, an aliquot of the cell suspension was removed and treated as described above for the preparation of glycopeptides. Figure 2 shows the glycopeptide profiles obtained in this experiment. It can be seen that at 0 time (no chase), the glycopeptide profile was very similar to that seen in control cells (see Fig. 1), with the radioactivity eluting in a rather broad peak between the $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ and $\text{Man}_9\text{GlcNAc}_2$ standards. In the presence of castanospermine little or no processing occurred, as shown by the remaining glycopeptide profiles of Fig. 2, and even after 90 min of chase, most of the radioactivity eluted from the Bio-Gel columns in the same area as in the control. This experiment suggested that castanospermine was inhibiting the processing of the oligosaccharide chains of

these glycoproteins, probably by inhibiting the removal of glucose residues. In this experiment, castanospermine did not inhibit the uptake or incorporation of [2- 3 H]mannose by the soybean cells, nor did it inhibit the incorporation of mannose into the lipid-linked oligosaccharides. In addition, the Bio-Gel P-4 profiles of the radioactive oligosaccharides isolated from the lipid-linked oligosaccharides were not altered by castanospermine.

In order to determine the specific step at which castanospermine was inhibiting the processing sequence, it was important to characterize the structures of the oligosaccharide portion of the glycopeptides that accumulated in the presence of this alkaloid. The glycopeptide peak from the 90-min chase in the presence of castanospermine (see Fig. 2) was pooled and separated into its various components on a

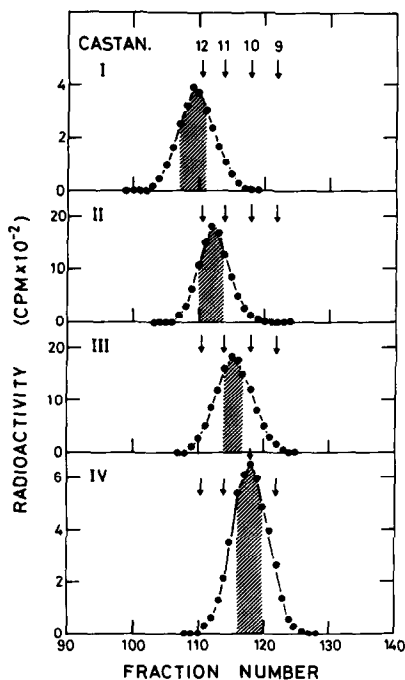


FIG. 3. Purification of individual glycopeptides from soybean cells incubated in castanospermine. Glycopeptides accumulated after a 90-min chase in castanospermine were separated on a long calibrated column of Bio-Gel P-4. Fractions under the hatched areas were pooled for further analysis. Standards shown by the arrows are the same as in Fig. 1.

long calibrated column of Bio-Gel P-4. Figure 3 shows that four glycopeptide peaks were isolated after several passages through this column, although there was some overlap between these peaks. After the last passage, the hatched areas of each peak were pooled for further analysis (see Fig. 3). The glycopeptide with the highest molecular weight (glycopeptide I) migrated somewhat faster than the $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ standard, while the smallest glycopeptide (glycopeptide IV) migrated with the $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$ standard (arrow corresponding to 10).

In order to characterize each of the glycopeptides shown in Fig. 3, they were treated with several different enzymes that have been shown to act on N-linked oligosaccharides. Thus, endoglucosaminidase H cleaves high-mannose and hybrid structures, but not complex chains, between the two internal GlcNAc residues to yield oligosaccharides having a single GlcNAc at the reducing terminus (17). The specificity of this enzyme requires that the α 1,6-linked mannose be substituted with another mannose residue, presumably in α 1,3-linkage (18). Jack bean α -mannosidase, on the other hand, can remove all of the α -linked mannose units from high-mannose chains, but apparently has difficulty cleaving the α 1,6-linked mannose from hybrid or glucose-containing oligosaccharides.

Each glycopeptide was digested with endoglucosaminidase H, and the products were rechromatographed on the calibrated column of Bio-Gel P-4 as shown in Fig. 4. In each of the profiles shown in the figure the original position of the glycopeptide (i.e., before treatment with endoglucosaminidase H) is shown by the arrows marked og. Thus, it can be seen that each glycopeptide was quite susceptible to digestion by endoglucosaminidase H as evidenced by the appearance of a new and slower migrating radioactive oligosaccharide resulting from the loss of the GlcNAc -peptide. Thus, following digestion, glycopeptide I gave a new peak that migrated with the $\text{Hexose}_{12}\text{GlcNAc}_1$ standard with a shoulder in the $\text{Hexose}_{11}\text{GlcNAc}_1$ position; glycopeptide II migrated with the

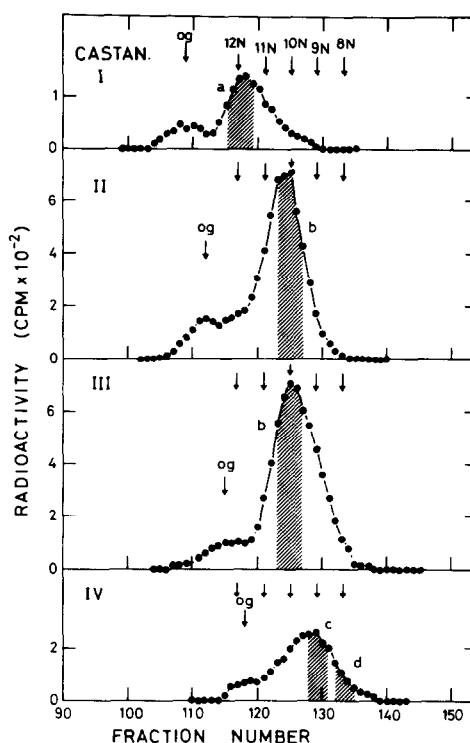


FIG. 4. Endoglucosaminidase H digestion of glycopeptides accumulated in castanospermine. Glycopeptides I, II, III, and IV from Fig. 3 were digested with endoglucosaminidase H and the reaction products were separated on a calibrated P-4 column. The arrows correspond to the following standards: 12N = $\text{Glc}_3\text{Man}_9\text{GlcNAc}_1$; 11N = $\text{Glc}_2\text{Man}_9\text{GlcNAc}_1$; 10N = $\text{Glc}_1\text{Man}_9\text{GlcNAc}_1$; 9N = $\text{Man}_9\text{GlcNAc}_1$; 8N = $\text{Man}_8\text{GlcNAc}_1$. og = the position of the original glycopeptide before endoglucosaminidase H treatment. An aliquot of each fraction was removed for determination of radioactivity.

$\text{Hexose}_{11}\text{GlcNAc}_1$ and $\text{Hexose}_{10}\text{GlcNAc}_1$ standards; glycopeptide III with the $\text{Hexose}_{10}\text{GlcNAc}_1$ standard; and glycopeptide IV with the $\text{Hexose}_{10}\text{GlcNAc}_1$ and $\text{Hexose}_9\text{GlcNAc}_1$ standards. Since these studies were done in cell culture, it is probably not surprising to find a mixture of glycopeptides, some of which have undergone partial processing even in the presence of castanospermine. However, the major oligosaccharide observed in these cells incubated with castanospermine was a $\text{Hexose}_{10}\text{GlcNAc}_1$ (hatched area labeled b in Fig. 4, profiles II and III; Note the difference in scales of radioactivity in the various

profiles). It was important to determine how many glucose residues were present in each of these oligosaccharides seen in Fig. 4 in order to determine the exact site of inhibition by this alkaloid.

Each of the oligosaccharides released by endoglucosaminidase H and shown by the hatched areas in Fig. 4 were pooled into fractions labeled a, b, c, and d, corresponding to Hexose₁₂GlcNAc₁, Hexose₁₀GlcNAc₁, Hexose₉GlcNAc₁, and Hexose₈GlcNAc₁. As indicated above, the Hexose₁₀GlcNAc₁ (fraction b) was by far the major oligosaccharide and accounted for more than 80% of the total radioactivity in the castanospermine-derived glycopeptides. Each of these oligosaccharides was digested exhaustively with an excess of jack bean α -mannosidase, and the incubation mixtures were then rechromatographed on the Bio-Gel P-4 column. Figure 5 shows the results of this experiment. The oligosaccharides corresponding to Hexose₁₀GlcNAc₁ (peak b), Hexose₉GlcNAc₁ (peak c), and Hexose₈GlcNAc₁ (peak d) all gave rise to a new major peak corresponding to the Hexose₇GlcNAc₁ standard, as well as to free mannose, following this enzymatic digestion. Thus, α -mannosidase digestion released 3, 2, or 1 mannose residues, respectively, from these oligosaccharides.

However, it can be seen from the profiles in Fig. 5 (b, c, d) that the newly formed peaks migrating with the Hexose₇GlcNAc₁ standard were not very sharp and had shoulders of higher-molecular-weight material (i.e., Hex₈GlcNAc₁). This finding suggested that these oligosaccharides were heterogeneous. The heterogeneity might be the result of either or both of the following: (i) the original oligosaccharides a, b, c, and d were not completely homogeneous since it is difficult to completely resolve oligosaccharides differing by only one hexose even on a long column of Bio-Gel P-4; and (ii) the α -mannosidase digestions may not have gone to completion since it is known that α -mannosidase works only slowly on the α 1,6-linked mannose residue when the α 1,3 branch is blocked by terminal glucose residues. Thus, in the digestions with b, c, and d, there is likely to be some

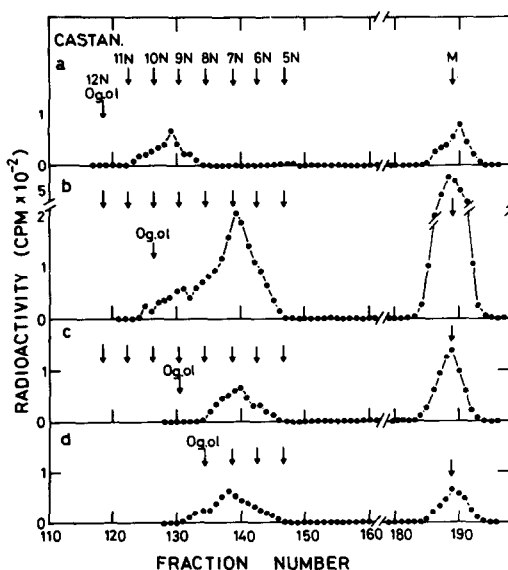


FIG. 5. α -Mannosidase digestions of castanospermine-induced oligosaccharides. Oligosaccharides (a, b, c, d) resulting from endoglucosaminidase H digestions as shown in Fig. 4 were treated with jack bean α -mannosidase and the digests were rechromatographed on Bio-Gel P-4. Standards are as shown in Fig. 4. Og = original position of endoglucosaminidase H-released oligosaccharide. M = mannose.

Hex₈GlcNAc₁ still present. Oligosaccharide a, which migrated like a Hexose₁₂GlcNAc₁, was also subjected to α -mannosidase digestion as seen in the upper profile of Fig. 5. In this case, the major peaks corresponded to a Man₉GlcNAc₁ and to free mannose. Some heterogeneity was also observed in this case since a shoulder of radioactivity was seen in the Hexose₁₀GlcNAc₁ area. These data suggest that some of the α -linked mannose units in peak a are either blocked by another substituent besides glucose, or are not susceptible to α -mannosidase for other reasons.

The Hexose₁₀GlcNAc₁ resulting from the castanospermine inhibition was subjected to complete methylation and after complete acid hydrolysis, the methylated mannose derivatives were identified by thin-layer chromatography. Fig. 6 (lower profile) shows the radioactive tracing of this thin-layer chromatogram. Radioactive bands were detected that corresponded to

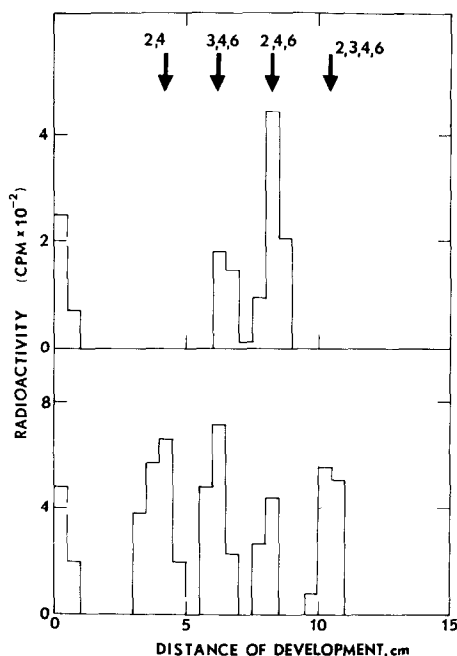


FIG. 6. Methylation analysis of [^3H]mannose-labeled Hexose $_{10}$ GlcNAc $_1$ (lower profile) and α -mannosidase-digested Hexose $_{10}$ GlcNAc $_1$ (i.e., Hexose $_7$ GlcNAc $_1$) (upper profile). Oligosaccharides were isolated from Bio-Gel P-4 columns and subjected to complete methylation. After isolation, the methylated oligosaccharides were hydrolyzed in acid and the methylated mannose derivatives were identified by thin-layer chromatography in benzene:acetone:water:ammonium hydroxide (50:200:3:1.5). Sections (0.5 cm) of the plate were scraped into vials and counted to determine the amount of radioactivity. Standard methylated mannose derivatives are 2,4 = 2,4-dimethylmannose; 3,4,6 = 3,4,6-trimethylmannose; 2,4,6 = 2,4,6-trimethylmannose; and 2,3,4,6 = 2,3,4,6-tetramethylmannose.

2,3,4,6-tetramethylmannose, 2,4,6-trimethylmannose, 3,4,6-trimethylmannose, and 2,4-dimethylmannose, indicating the presence of terminal mannose, 3-linked mannose, 2-linked mannose, and 3,6-linked mannose. Based on the known structure of the Glc $_3$ Man $_9$ GlcNAc $_2$ oligosaccharide (6-8), the presence of 3-linked mannose is suggestive of a mannose residue carrying a glucose substitution. Thus, these mannose derivatives are what would be expected from a glucose-containing oligosaccharide such as a Glc $_3$ Man $_7$ GlcNAc $_1$ structure. Unfortunately in this experi-

ment, it is not meaningful to compare the ratios of radioactivity in the various mannose derivatives since the glycopeptides were derived from short-term incubations (i.e., 15 min) with [^3H]mannose. Thus, it is likely that the various mannose residues are not equally labeled and that equilibration of oligosaccharide labeling has not occurred.

The Hexose $_7$ GlcNAc $_1$ structure resulting from exhaustive α -mannosidase digestion was also subjected to complete methylation and the methylated mannose derivatives were examined by thin-layer chromatography as shown in Fig. 6 (upper profile). In this case, only two radioactive peaks were detected that corresponded to 3,4,6-trimethylmannose and 2,4,6-trimethylmannose. The absence of terminal mannose indicates that all of the unsubstituted mannose residues have been removed by the α -mannosidase to leave a Hexose $_7$ GlcNAc $_1$ which is most likely to be a Glc $_3$ Man $_4$ GlcNAc $_1$ structure. The absence of any 3,6-linked mannose in this oligosaccharide also supports such a structure.

Finally, the [^3H]mannose-labeled Hexose $_{10}$ GlcNAc $_1$ from the castanospermine-derived glycopeptides was incubated with a rat liver particulate enzyme preparation that has been shown to contain the processing glucosidases (19). If the Hexose $_{10}$ GlcNAc $_1$ does contain three glucose residues, then one would expect to see this oligosaccharide trimmed to smaller oligosaccharides having 1, 2, or 3 fewer hexose residues. Following the incubation with particulate enzyme, the reaction mixture was analyzed on the Bio-Gel P-4 column. This incubation gave rise to three new radioactive peaks that corresponded to Hexose $_9$ GlcNAc $_1$, Hexose $_8$ GlcNAc $_1$, and Hexose $_7$ GlcNAc $_1$ standards (data not shown). The Hexose $_9$ GlcNAc $_1$ and Hexose $_8$ GlcNAc $_1$ peaks were present in greatest amounts with considerably less of the Hexose $_7$ GlcNAc $_1$ structure. Apparently during this incubation period (6 h), no radioactive mannose was released since no radioactivity was observed in the mannose area of this column. Thus, this data taken together with the methylation data indicate that

the major structure that accumulates in soybean cell glycoproteins in the presence of castanospermine is a $\text{Glc}_3\text{Man}_7\text{GlcNAc}_2$.

DISCUSSION

The data presented in this paper show that the indolizidine alkaloid castanospermine inhibits the processing of the asparagine-linked oligosaccharides in suspension-cultured soybean cells. The major oligosaccharide that accumulated in the glycoproteins of inhibited cells was a $\text{Hexose}_{10}\text{GlcNAc}_2$. Since this oligosaccharide was shown to contain three glucose residues, castanospermine must inhibit the removal or trimming of the outermost glucose. Thus, even though the glucoses have not been removed, the glycoproteins can still undergo some processing with the removal of mannose residues from the 1,6 branch to give a series of oligosaccharides partially characterized as $\text{Glc}_3\text{Man}_7\text{GlcNAc}_2$, $\text{Glc}_3\text{Man}_6\text{GlcNAc}_2$, and $\text{Glc}_3\text{Man}_5\text{GlcNAc}_2$. In animal cells, castanospermine has also been shown to inhibit processing at the glucosidase I stage and to give rise to glycoproteins having mostly $\text{Glc}_3\text{Man}_7\text{GlcNAc}_2$ structure (13). In animal cells, the mannosidase that removes the $\alpha 1,3$ - and $\alpha 1,6$ -linked mannoses from the 1,6 branch (mannosidase II) requires the presence of a terminal GlcNAc on the $\alpha 1,3$ -linked mannose that is attached to the β -linked mannose (20, 21). Since the 1,3 branch still contains terminal glucose residues in the presence of castanospermine, mannosidase II cannot act on these oligosaccharides and trimming stops at the $\text{Glc}_3\text{Man}_7\text{GlcNAc}_2$ stage. Thus, the finding of some $\text{Glc}_3\text{Man}_6\text{GlcNAc}_2$ and $\text{Glc}_3\text{Man}_5\text{GlcNAc}_2$ in the plant glycoproteins suggests that the processing mannosidases in soybean cells have different specificities than those in animal cells.

Inhibitors of glycoprotein biosynthesis and processing can be valuable tools to study the reactions involved in the formation of various oligosaccharide structures and also to examine the role(s) of various oligosaccharide structures in gly-

coprotein function. The first inhibitor of glycoprotein processing to be described was the plant indolizidine alkaloid swainsonine (22). Swainsonine (1,2,8-trihydroxyoctahydroindolizine) was initially isolated from the Australian plant *Swainsona canescens* (23) and shown to be a potent inhibitor of lysosomal and jack bean α -mannosidase (24). It was later isolated from spotted locoweed that grows in the southwestern United States (25), and from the fungus *Rhizoctonia leguminicola* (26). Initial studies on the effect of swainsonine on cultured mammalian cells (22) or on influenza virus-infected canine kidney cells (27) showed that this alkaloid prevented the formation of complex types of oligosaccharides and gave rise to glycoproteins whose oligosaccharides were almost entirely susceptible to digestion by endoglucosaminidase H. Subsequent studies in Touster's laboratory (28) showed that swainsonine specifically inhibits mannosidase II and *in vitro* studies with this inhibitor gave rise to hybrid-type oligosaccharides. Such hybrid-type oligosaccharides have also been found in a variety of cultured cells as a result of swainsonine inhibition (29-31).

Several other inhibitors of glycoprotein processing have recently been described and the results with these compounds are somewhat analogous to those with the plant alkaloids. Deoxynojirimycin is an antibiotic that was found to be an inhibitor of yeast and pancreatic α -glucosidase. This compound inhibited both of the partially purified glucosidases of yeast and also inhibited calf pancreas microsomal glucosidases (32). It also inhibited calf liver microsomal oligosaccharide-glucosidases (33). These enzymes are involved in the trimming of the three glucose residues from the $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$. At 5 mM concentrations, deoxynojirimycin greatly decreased the proportion of radioactive sugars incorporated into the complex types of oligosaccharides in IEC-6 intestinal epithelial cells, and rendered the high-mannose oligosaccharides less susceptible to the action of α -mannosidase (32). Thus, these oligosaccharides may be similar to those formed in the presence of castano-

spermine, but they have not yet been completely characterized.

Bromoconduritol is another recently described inhibitor of glycoprotein processing. This compound was reported to inhibit the processing of the innermost glucose residue and to give rise to Glc₁Man₉GlcNAc₂ and Glc₁Man₈GlcNAc₂ structures (34). The inhibition by bromoconduritol occurred both in intact cells and in microsomal preparations. Unfortunately, bromoconduritol is quite unstable and has a half-life in water of only 16 min at 37°C and pH 7.3. This, and the fact that millimolar concentrations are required for inhibition, will limit the use of this material. Nevertheless, the isolation and utilization of various inhibitors of glycoprotein processing should greatly benefit our understanding of the biosynthetic pathway and also allow a detailed study of the role of carbohydrate in glycoprotein function.

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